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14. ABSTRACT
In an effort to gain a better understanding of the consequence of deregulated CDK4 activity *in vivo*, we generated knock-in transgenic mice that expresses a tumor derived mutant form of CDK4 (r24c). Interestingly, these mice are very prone to develop tumors of different cellular origins, including breast tumors. Since the p53 tumor suppressor pathway is an inhibitor of oncogene induced transformation and is inactivated in a high percentage of mouse and human tumors, we decided to determine if inactivation of the p53 pathway is an important step in CDK4 mutant induced tumorigenesis. Our investigation revealed that MDM2 is overexpressed in 3 out of 10 breast tumors from these transgenic mice and we have found no evidence that the p53 pathway is inactivated in these tumors that arise in these animals. MDM2 has oncogenic properties when overexpressed and high amounts of the protein are present in human cancers. It is generally accepted that MDM2 promotes tumorigenesis by directly blocking the function of the p53 tumor suppressor. However, recent studies indicate that MDM2 promotes cell proliferation by mechanisms that are independent of inactivating p53, including by inhibiting the anti proliferative activity of Transforming Growth factor-β1 (TGF β1) in breast tumors. Considering that we have found no evidence for loss of p53 function by p53 gene mutation or loss of $p19^{ARF}$ expression, the scope of this proposal is to address if MDM2 overexpression cooperates with mutant CDK4 in mammary cell transformation and tumorigenesis by affecting p53 independent proliferation control

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Introduction

In an effort to gain a better understanding of the consequence of deregulated CDK4 activity *in vivo,* we generated knock-in transgenic mice that expresses a tumor derived mutant form of CDK4 (r24c). Interestingly, these mice are very prone to develop tumors of different cellular origins, including breast tumors. Since the p53 tumor suppressor pathway is an inhibitor of oncogene induced transformation and is inactivated in a high percentage of mouse and human tumors, we decided to determine if inactivation of the p53 pathway is an important step in CDK4 mutant induced tumorigenesis. Our investigation revealed that MDM2 is overexpressed in 3 out of 10 breast tumors from these transgenic mice and we have found no evidence that the p53 pathway is inactivated in these tumors that arise in these animals. MDM2 has oncogenic properties when overexpressed and high amounts of the protein are present in human cancers. It is generally accepted that MDM2 promotes tumorigenesis by directly blocking the function of the p53 tumor suppressor. However, recent studies indicate that MDM2 promotes cell proliferation by mechanisms that are independent of inactivating p53, including by inhibiting the anti proliferative activity of Transforming Growth factor-β1 (TGF β1) in breast tumors. Considering that we have found no evidence for loss of p53 function by p53 gene mutation or loss of $p19^{ARF}$ expression, the scope of this proposal is to address if MDM2 overexpression cooperates with mutant CDK4 in mammary cell transformation and tumorigenesis by affecting p53 independent proliferation control pathways.

Body

In the first task outlined in the approved Statement of Work we set out to determine if there is a higher incidence of breast tumors in CDK4 mutant mice that overexpress MDM2 when compared to CDK4 mutant mice that lack p53. To address this hypothesis we obtained, genotyped and established mouse colonies of MDM2 transgenic mice as well as p53 knockout mice. Genomic DNA was isolated from the tail snips of mouse pups and MDM2 southern blots were performed to identify those mice that expressed the greatest copy number of MDM2 gene. Mice harboring the p53 knockout alleles were identified using a PCR approach. Numerous reports have demonstrated that different genetic backgrounds can have a profound influence on tumor spectra that arise in mouse models. This posed a significant concern for our tumor development studies since all three mice, MDM2, p53 and CDK4 r24c, were on three different genetic backgrounds. Therefore, before generating mice that 1) overexpress MDM2 and are homozygous for the CDK4R24C mutation 2) overexpress MDM2 and contain wild type CDK4 alleles, 3) contain normal levels of MDM2 and are homozygous for the CDK4 r24c mutation, 4) p53 null and homozygous for the CDK4 r24c mutation and 5) p53 null we back crossed all three mouse strains for 6 successive generation onto the same BL6 genetic background. Currently, we are establishing mice colonies for each respective compound mouse combination.

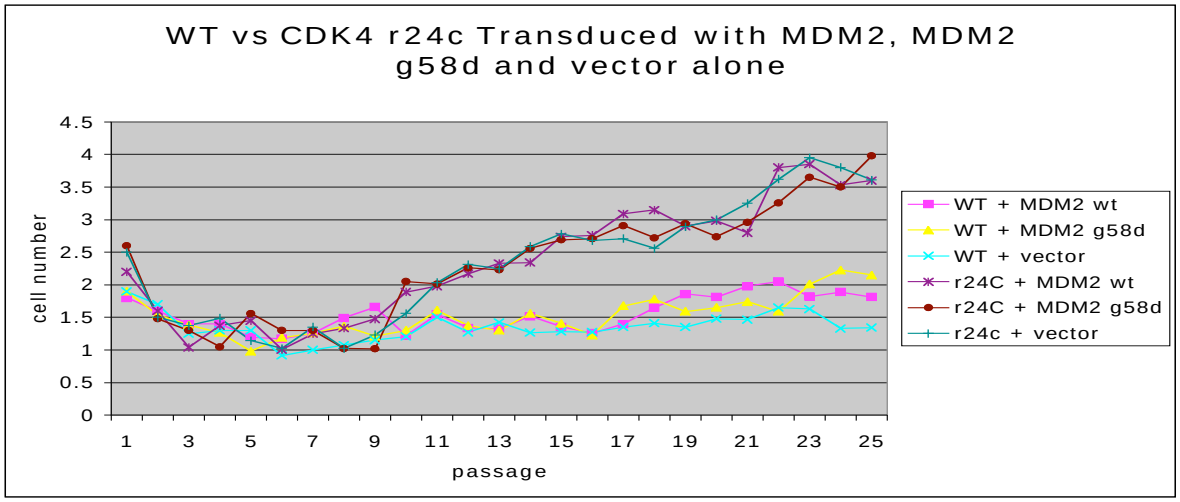
In the second task from the Statement of Work we set out to determine if overexpression of a p53-binding deficient MDM2 mutant immortalizes and/or transforms primary mammary epithelia cells (MECs) harboring a CDK4 mutation at a higher efficiency when compared to MECs that contain wild type CDK4. To address this hypothesis we harvested MECs from six-week-old CDK4 r24c and wild type mice. Early passage MECs from CDK4 mutant mice and wild type controls were transduced with

recombinant retrovirus encoding wt MDM2, the p53-binding deficient MDM2 mutant g58d or empty vector control. The phoenix packing cell line was transfected by calcium phosphate with the various recombinant retroviral constructs. High titer supernatants were placed on the MECs for 24 hours. The media was removed after retroviral infection, cells were rinsed with PBS, and new growth media was added. The media was changed every 3 to 4 days. Immunoblotting was performed to insure proper overexpression of the transduced genes. After 2.5 weeks, foci were stained with crystal violet and the number of foci containing cells of a transformed phenotype was counted. This transformation assay revealed there was no difference in the number foci formed after transducing wild type and CDK4 mutant MECs with MDM2 wt, the p53-binding deficient MDM2 mutant or vector alone. See table 1.

Table 1. Number of foci/plate mean

	CDK4 wt	CDK4 r24c/r24c
MDM2 wt	0	0
MDM2 g58d	0	0
Vector	0	0

For the immortalization studies, three separate early passage MECs from wt and CDK4 mutant mice were transduced with MDM2 wt, MDM2 g58d and empty vector alone recombinant retroviruses as described above. Cells were propagated in F12 growth for 25 passages according to the 3T3 protocol. 3×10^5 cells were cultured in 100 X 15 mm plates. Three days later the total number of cells were counted and 3×10^5 cells were re-plated which constituted one passage. This process was continued for 25 successive passages and the cumulative increase in cell number was calculated. The results from plotting the values of this 3T3 transformation assay revealed that overexpressing either wt MDM2 or the p53 binding deficient mutant of MDM2 in MECs does not cooperate with mutant CDK4 in allowing cells to escape cellular senescence. Both the wild type and CDK4 mutant MECs experienced and underwent a very comparable crisis period. The escape from cellular senescence and increased growth kinetics in the CKD4 r24c MECs can be attributed to the cells harboring the CDk4 mutation.



Key Research Accomplishments:

- Maintained and successfully genotyped MDM2 transgenic and p53 knockout mice
- Backcrossed MDM2, p53 and CDK4 r24c mice into the same genetic background
- The following compound mouse colonies are currently being generated:
    1) Mice that overexpress MDM2 and are homozygous for the CDK4R24C mutation
    2) Mice that overexpress MDM2 and contain wild type CDK4 alleles
    3) Mice that contain normal levels of MDM2 and are CDK4 r24c homozygous
    4) Mice that are p53 null and homozygous for the CDK4R24C mutation
    5) Mice that are p53 null
- Determined that overexpression of a p53-binding deficient MDM2 mutant DOES NOT immortalize and/or transform primary MECs harboring a CDK4 mutation at a higher efficiency when compared to MECs that contain wild type CDK4.

Reportable Outcomes: None.

Conclusions:

All the experimental objectives in the second task of the Approved Statement of work were accomplished. The foci transformation assay revealed there was no difference in the number foci formed after transducing wild type and CDK4 mutant MECs with retroviruses expressing MDM2 wt or the p53-binding deficient MDM2 mutant. The results from plotting the values of this 3T3 transformation assay revealed that overexpressing either wt MDM2 or the p53 binding deficient mutant of MDM2 in MECs does not cooperate with mutant CDK4 in allowing cells to escape cellular senescence. When taken together, these results suggest that MDM2 and mutant CDK4 do not cooperate in promoting cellular immortalization of mammary epithelial cells *in vitro*. Since we did not find any synergistic affect between MDM2 and the CDK4 mutant using these *in vitro* assays, this may suggest that the cooperation between MDM2 and mutant CDK4 in cellular immortalization and transformation occurs under *in vivo* selective pressures or is cell type specific. These possibilities will be addressed using the compound mouse crosses described in the first task of the approved Statement of Work.

References:

Appendices: None.